Microplate Alamar Blue Assay for *Staphylococcus epidermidis* Biofilm Susceptibility Testing

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Biofilms are at the root of many infections largely because they are much more antibiotic resistant than their planktonic counterparts. Antibiotics that target the biofilm phenotype are desperately needed, but there is still no standard method to assess biofilm drug susceptibility. *Staphylococcus epidermidis* ATCC 35984 biofilms treated with eight different approved antibiotics and five different experimental compounds were exposed to the oxidation reduction indicator Alamar blue for 60 min, and reduction relative to untreated controls was determined visually and spectrophotometrically. The minimum biofilm inhibitory concentration was defined as ≤50% reduction and a purplish well 60 min after the addition of Alamar blue. All of the approved antibiotics had biofilm MICs (MBICs) of >512 µg/ml (most >4,096 µg/ml), and four of the experimental compounds had MBICs of ≤128 µg/ml. The experimental aaptamine derivative hystatin 3 was used to correlate Alamar blue reduction with 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) reduction and viable counts (CFU/ml) for *S. epidermidis* ATCC 35984, ATCC 12228, and two clinical isolates. For all four strains, Alamar blue results correlated well with XTT ($r = 0.83$ to 0.97) and with CFU/ml results ($r = 0.85$ to 0.94). Alamar blue's stability and lack of toxicity allowed CFU/ml to be determined from the same wells as Alamar blue absorbances. If the described method of microplate Alamar blue biofilm susceptibility testing, which is simple, reproducible, cost-effective, nontoxic, and amenable to high throughput, is applicable to other important biofilm forming species, it should greatly facilitate the discovery of biofilm specific agents.

Given the tremendous clinical importance of biofilms, it is somewhat surprising that there is no standard method for investigating the drug susceptibility of bacterial biofilms. Several methods are available but are limited by long processing times, incompatibility with high throughput, expensive reagents or equipment, or the fact that the method measures mass instead of viability (2, 4, 7, 13, 14, 24, 25). For bacteria, a common method of assessing susceptibility is to quantitate the mass of biofilms by crystal violet or safranin staining, followed by extraction of bound dye with a solvent and measurement of absorption (6, 24). This method provides no information about viability. Another common method of assessing bacterial biofilm susceptibility is to disrupt the biofilm by sonication, vortexing, or scraping, followed by dilution plating for determination of CFU/ml (27, 28, 31). This method has serious limitations; biofilm clumps can be difficult to dissociate into single-cell suspensions for plate counting, it is extremely laborious, and antibiotic carryover is a concern. For fungi, the most common method is an XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] reduction assay (1, 8, 25), and this method has also been used for bacterial biofilms (1). While XTT reduction does measure metabolic activity, it requires the addition of an electron coupling reagent. In addition, the reagents are toxic to the investigator and cells of

interest, which would prevent concurrent determination of viability and long-term kinetic studies.

Clearly, there is a need for an easy, cost-effective, reproducible assay of biofilm susceptibility that measures viability. The redox indicator Alamar blue (AB) has been used extensively in planktonic bacterial susceptibility assays (5, 10), planktonic fungal susceptibility assays (23, 30), and mammalian cell culture cytotoxicity assays (3, 18). The AB assay is a simple, one-step procedure, quite amenable to high throughput, whereby metabolic activity results in the chemical reduction of AB. Alamar blue is reduced by $FMNH_2$, $FADH_2$, NADH, NADPH, and the cytochromes (product literature, Trek Diagnostic Systems). Alamar blue both fluoresces and changes color in response to chemical reduction, and the extent of the conversion is a reflection of cell viability (product literature, Trek Diagnostic Systems). Continued growth maintains a reduced environment while inhibition of growth maintains an oxidized environment. Alamar blue is water soluble, so the washing/fixation/extraction steps required in other commonly used cell proliferation assays are not required. Data may be collected with the naked eye, or for increased sensitivity, with either fluorescence-based or absorbance-based instruments. Alamar blue is also nontoxic to both the investigator and to the cells of interest, so it is safe to work with, easily disposed of, and less likely to interfere with normal metabolism in test cells. In addition, AB is stable, so long incubations are possible, as are kinetic studies.

Most catheter-associated nosocomial infections are caused by coagulase-negative staphylococci, and *Staphylococcus epi-*

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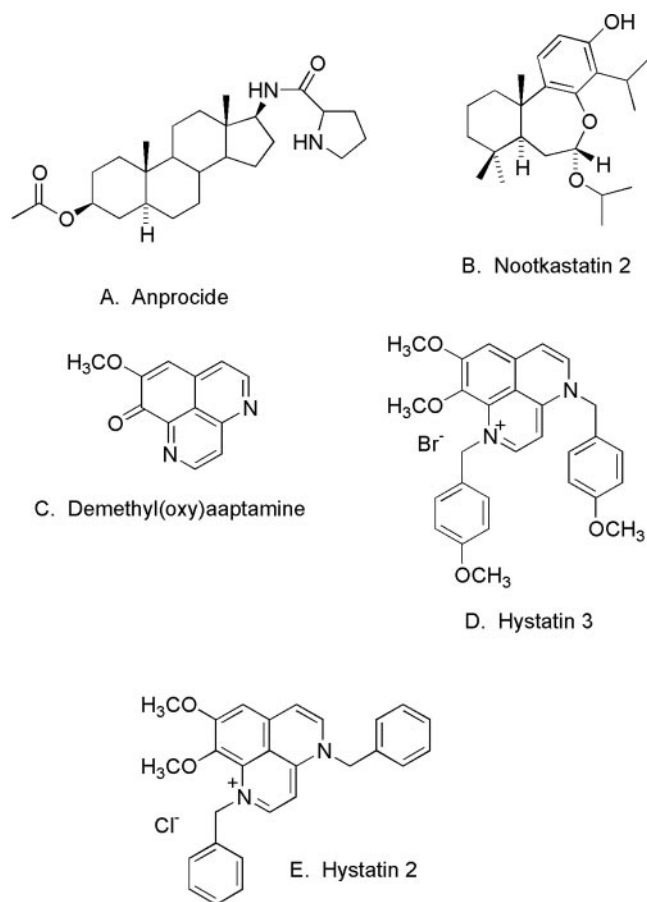
FIG. 1. Structures of experimental compounds. (Figure provided by F. Hogan and published with permission.)

dermidis is responsible for 50 to 70% of reported cases (26). Biofilm-positive *S. epidermidis* RP62A (ATCC 35984), isolated from a patient with intravascular catheter-associated sepsis (9), was used to determine if AB could be applied to bacterial biofilm drug susceptibility screening.

MATERIALS AND METHODS

Strains. *S. epidermidis* ATCC 35984 (RP62A), ATCC 12228, and two clinical isolates (S67166 from a central venous catheter, X64787 from an endotracheal tube; Phoenix Children's Hospital, Phoenix, AZ) were maintained on Mueller-Hinton agar (MHA) at 35°C. To confirm biofilm formation, strains were grown in cation-adjusted Mueller-Hinton II broth (MHIIB) for 24 h in flat bottom, polystyrene, non-tissue-culture-treated microtiter plates. Ten μl of a 1% solution of Congo red was added to each well, and the plates were shaken briefly and incubated 10 min at room temperature. Liquid was removed from the wells, and the wells were gently washed with saline. Stained biofilms were visualized by phase-contrast microscopy. *S. epidermidis* ATCC 12228, S67166, and X64787 were compared to the biofilm-positive strain ATCC 35984 and the biofilm "negative" mutant ATCC 49461.

Antibiotics and experimental compounds. Bacitracin, vancomycin, gentamicin, rifampin, nitrofurazone, and enrofloxacin were obtained from ICN. Ceftriaxone and oxacillin were from Sigma. Rifampin, nitrofurazone, and enrofloxacin were dissolved in sterile dimethyl sulfoxide, and the remaining antibiotics were dissolved in sterile H_2O. Experimental compounds (Fig. 1) were dissolved in sterile dimethyl sulfoxide or MeOH.

Alamar blue biofilm susceptibility assay. Alamar blue (Trek Diagnostic Systems) was aliquoted and stored at −80°C. Prior to each experiment, AB was brought to room temperature and vortexed. Exposure of AB to light was mini-

mized throughout the experiments. Isolated colonies from 18 to 22 h MHA plates were used to prepare inocula. Assays were performed in flat-bottom, polystyrene, non-tissue-culture-treated microtiter plates containing 5×10^5 CFU/ml in MHIIB media, with final well volumes of 100 μl. Plates were incubated at 37°C without shaking. Twofold dilutions of drugs in cation-adjusted MHIIB were prepared external to the plates, with approved antibiotics diluted 4,096 to 64 μg/ml and experimental drugs diluted 64 to 0.5 μg/ml. After 24 h, 50 μl was removed from all experimental and control wells, and 50 μl of the appropriate drug dilution was added. Biofilms were exposed to drugs for 20 h at 37°C without shaking. After 20 h, 5 μl AB was added to the wells (105 μl total volume) and the plates were shaken gently and incubated for 1 h at 37°C. Plates were gently shaken again, and absorbances at 570 nm and 600 nm were obtained in a Perkin-Elmer Wallac Victor[3] microplate reader. For experiments with multiple time points, plates were kept in a 37°C incubator between absorbance readings. Controls included media alone, media plus AB, media plus AB plus drug dilution, and cells plus media plus AB. Biofilm susceptibility experiments were performed a minimum of two times. Percent reduction of AB was calculated using the manufacturer's formula, with replacement of their negative control, which contains only media plus AB, with a more robust negative control, media plus AB plus a drug concentration equal to each experimental well:

$$\frac{(\varepsilon_{ox})\lambda_2 A\lambda_1 - (\varepsilon_{ox})\lambda_1 A\lambda_2}{(\varepsilon_{red})\lambda_1 A'\lambda_2 - (\varepsilon_{red})\lambda_2 A'\lambda_1} \times 100$$

where ε_{ox} = molar extinction coefficient of Alamar blue oxidized form (blue), ε_{red} = molar extinction coefficient of Alamar blue reduced form (pink), A = absorbance of test wells, A' = absorbance of negative control well, λ_1 = 570 nm, and λ_2 = 600 nm.

Alamar blue planktonic susceptibility assay. Planktonic susceptibility testing of *S. epidermidis* was performed by the reference broth microdilution assay outlined by NCCLS (17), using round-bottom, polystyrene, non-tissue culture treated microtiter plates and cation-adjusted MHIIB. After 19 h, visual MICs were recorded, 5 μl of AB was added, and the plates were shaken and incubated at 37°C. After 1 h in the presence of AB, well contents were removed and transferred to a flat-bottom plate for absorbance readings at 570 nm and 600 nm. Assays were performed at least twice, and the average % reduction was used to determine the MIC. The AB MIC was defined as the lowest drug concentration resulting in ≤50% reduction of AB and a purplish well 60 min after the addition of AB.

Biofilm XTT reduction assay. The sodium salt of XTT (Sigma) was dissolved in phosphate-buffered saline to 1 mg/ml. Menadione (Sigma) was dissolved in acetone to 1 mM. The XTT/menadione reagent was prepared fresh prior to each assay and contained 12.5 parts XTT/1 part menadione. Biofilms were established and drug treated as for the AB assay. After 20 h of drug treatment, 25 μl XTT/menadione was added to the wells and the plates were gently shaken. After 1 h at 37°C, plates were again shaken gently and absorbance at 490 nm was obtained in the microplate reader. For experiments with multiple time points, plates were kept in a 37°C incubator between absorbance readings. Controls included media alone, media plus XTT/menadione, media plus XTT/menadione plus a drug concentration equal to each experimental well (negative control), and cells plus media plus XTT/menadione (positive control). Percent formazan produced was calculated using the following formula: (experimental well absorbance − negative control absorbance)/positive control absorbance × 100.

Biofilm CFU/ml assay. CFU/ml were obtained from the same wells used to obtain biofilm AB absorbances as follows. After drug treating and obtaining AB absorbance readings (several minutes/plate), wells were scraped thoroughly, with particular attention to well edges. Well contents were removed, placed in 1 ml sterile saline, centrifuged at 10,000 rpm for 10 min, the supernatant was removed, and the cells were washed twice with saline. Cells were resuspended in 100 μl saline, diluted in saline blanks, and plated on MHA. Colonies were counted after 24 h at 35°C. CFU/ml were also determined prior to drug treatment at 24 h; these biofilms were washed in the same manner as drug-treated biofilms prior to dilution plating.

Correlation of Alamar blue reduction to XTT reduction and CFU/ml. For correlation experiments, AB, XTT, and CFU/ml assays were performed the same day using a single inoculum. Experiments were repeated at least twice. Pearson's two-tailed correlations were calculated with Prism 4 software using averaged data from the entire range of drug concentrations.

RESULTS AND DISCUSSION

Confirmation of biofilm formation. Biofilms covered with matrix material were evident in Congo red-stained phase-con-
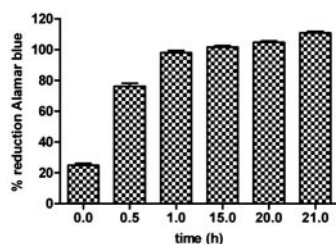
FIG. 2. Percent reduction of Alamar blue over time for untreated *S. epidermidis* ATCC 35984 biofilms.

trast micrographs of all four strains, with somewhat less cellular material and matrix in the "negative" control ATCC 49461 (data not shown).

Alamar blue assay standardization. A variety of parameters for the AB assay were standardized at the outset using *S. epidermidis* ATCC 35984. The goal was to make the biofilm susceptibility assay as similar to the NCCLS planktonic susceptibility assay (17) as possible. Briefly, a starting inoculum of 5×10^5 CFU/ml (same as NCCLS planktonic inoculum) was selected from a range of cell densities that yielded biofilm AB reduction in the linear range (data not shown). The supplier of AB, Trek Diagnostic Systems, recommends a volume of AB that is 10% of the well volume. We found that 5 μl (in 105 μl total well volume) gave biofilm AB reduction values similar to larger volumes (data not shown), thus reducing assay costs further. Drug treatment times for bacterial biofilms vary greatly in the literature (12, 16, 29). In the present study, treatment duration was 20 h. The endpoint of AB reduction was another critical parameter. In order to make the assay as efficient as possible, the shortest possible endpoint was determined. Alamar blue was added to 24-h biofilms, and percent reduction over time was determined (Fig. 2). In these untreated 24-h biofilms, AB in wells turned from purplish to bright pink within 30 min and remained this color at least 21 h (Fig. 2). Next, 24-h biofilms were treated for 20 h with nitrofurazone or oxacillin. Alamar blue was then added, and absorbance was determined 30 to 90 min after the addition of AB (Fig. 3). The susceptibility pattern was clear at 60 min, so this time point was chosen as the endpoint for absorbance readings in all experiments. Oxacillin (Fig. 3B) is colorless, and thus susceptibility could be determined visually. At 30 min, all wells were purplish/pink, and at 60 and 90 min, all wells were pink. Nitrofurazone (Fig. 3A) is colored, and thus susceptibility could only be determined spectrophotometrically.

An important consideration in biofilm susceptibility testing is the high drug concentrations required to eradicate biofilms. As such, interactions between AB and drugs were considered. In microtiter wells containing only drug and AB, percent reduction sometimes exceeded 20% (data not shown). The manufacturer's formula for determining percent reduction of AB (see Materials and Methods) does not take AB/drug interactions into account. Thus, we replaced their negative control, which contains only AB and media, with negative controls containing these reagents in addition to drug concentrations equal to experimental wells (see Materials and Methods).

Alamar blue biofilm susceptibility assay. Prior to determining biofilm susceptibility, AB MICs for a variety of drugs with

different mechanisms were determined for planktonically grown organisms (Table 1). Visual and AB MICs for planktonically grown strains were generally identical or within one twofold dilution (data not shown). MICs and MBICs were defined as the lowest drug concentration resulting in ≤50% reduction of AB and a purplish well 60 min after the addition of AB. Alamar blue MBICs increased at least sevenfold relative to planktonic AB MICs (Table 1). These data are consistent with previous reports of many-fold increases in drug resistance of biofilm versus planktonic-grown strains (11). For colorless compounds, wells below the MBIC remained pink for a minimum of 60 min and wells at and above the MBIC remained purplish for a minimum of 60 min. In cases where plates were examined 24 to 72 h later, color remained stable. Thus, for noncolored drugs, MBICs can be determined visually or spectrophotometrically, while for colored drugs MBICs should be determined spectrophotometrically (also true for XTT). For colored compounds, only the percent reduction portion of the definition was used. For approved antibiotics, all wells containing colorless antibiotics, gentamicin, bacitracin, vancomycin, enrofloxacin, ceftriaxone, and oxacillin, were pink at 60 min, as were untreated controls. These colors remained stable for at least 72 h at 4°C. Nitrofurazone visibly precipitated at concentrations of >512 μg/ml, and rifampin visibly precipitated at >1,024 μg/ml. The experimental compounds anprocide [3β-acetoxy-17β-(L-prolyl)amino-5α-androstane], a synthetic steroidal amide (Fig. 1A) (22), nootkastatin 2, a diterpene from the Alaskan yellow cedar *Chamaecyparis nootkatensis* (21) (Fig. 1B), demethyloxyaaptamine, from the marine sponge *Hymeniacidon* sp. (20) (Fig. 1C), and two semisynthetic quaternary ammonium aaptamine derivatives, hystatin 3 and hystatin 2 (19) (Fig. 1D and E), were effective at lower concentrations than the approved antibiotics (Table 1). Anprocide and nootkastatin 2 are colorless, and thus susceptibility could also be determined visually. At 60 min, wells containing anprocide at 512 to 128 μg/ml were purplish; wells containing lower dilutions were all pink. Wells containing nootkastatin 2 at 512 to 64 μg/ml were purplish at 60 min; wells containing lower dilutions were all pink.

Should AB be developed as a standard method of biofilm susceptibility testing, it would be useful to have consensus on the definition of the MBIC. While the current study used a value of ≤50% reduction, much more data from a variety of labs will be necessary to define the most broadly applicable cutoff.

XTT assay standardization. Reduction of tetrazolium salts is a commonly used method of determining microbial and mammalian cell viability. As with the AB biofilm susceptibility assay, a variety of parameters for the XTT biofilm susceptibility assay were standardized. Biofilms were established exactly as for the AB. assay. At 2, 5, 10, and 25 μl XTT/menadione (in 100 μl total well volume), absorbance was detectable and unchanged from 1 to 4 h (data not shown). However, only the 25-μl volume allowed ready visualization of the orange-colored formazan product. Since absorbance remained stable from 1 to 4 h, absorbance at 490 nm was determined 1 h after the addition of 25 μl XTT-menadione. As with AB, considerable drug/XTT interaction was noted. Therefore, for all experiments, controls contained the same drug concentration as experimental wells, and absorbance of these controls was subtracted from
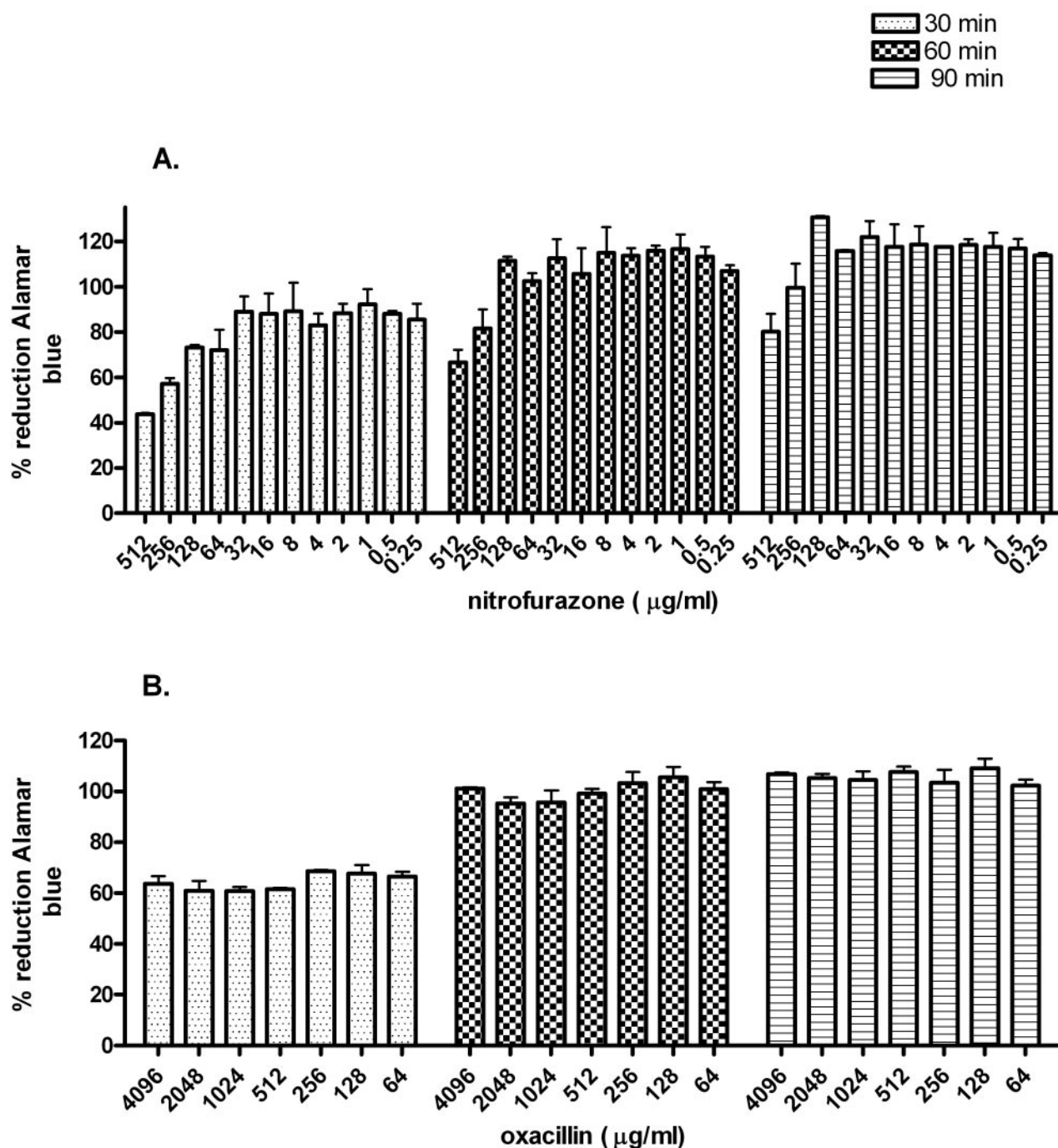
FIG. 3. Percent reduction of Alamar blue at 30, 60, and 90 min for *S. epidermidis* ATCC 35984 biofilms treated for 20 h with twofold dilutions of nitrofurazone (A) or oxacillin (B).

the experimental absorbance (see Materials and Methods). Similar to AB, susceptibility could only be determined spectrophotometrically for colored compounds. Although the formazan product is supposed to be water soluble and readily diffuse out of cells, we and others (15) have noted clumps of orange-stained material retained in biofilms after shaking. In contrast, with the AB method, microtiter wells containing biofilms have a very homogeneous pink or blue color after shaking.

Correlation of AB reduction with XTT reduction and CFU/ml. To help validate the described AB method, AB assays were performed in parallel with XTT reduction assays, and CFU/ml were obtained from the same wells as AB absorbances. Biofilms of two type strains and two clinical isolates of *S. epidermidis* were established overnight and treated with hystatin 3 for 20 h. Alamar blue or XTT/menadione were added, and after 1 h, absorbances were obtained and wells were scraped for

TABLE 1. Alamar blue MICs and MBICs[a] of approved and experimental drugs against planktonic- and biofilm-grown *S. epidermidis* ATCC 35984

Drug	Planktonic MIC (μg/ml)	Biofilm MBIC (μg/ml)
Gentamicin	16	>4,096
Bacitracin	32	>4,096
Nitrofurazone	4	>512
Vancomycin	2	>4,096
Enrofloxacin	1	>4,096
Ceftriaxone	32	>4,096
Oxacillin	8	>4,096
Rifampin	4	>1,024
Anprocide	16	128
Nootkastatin 2	8	64
Demethyloxyaaptamine	1	>512
Hystatin 3	2	64
Hystatin 2	0.5	128

[a] MIC or MBIC defined as the lowest drug concentration resulting in $\leq$50% reduction of AB (average of two experiments) and a purplish well 60 min after addition of AB.

dilution plating (Fig. 4). There was very little biofilm growth from 24 h (Fig. 4, arrows) to 44 h (Fig. 4, 0 μg/ml). As evident in Fig. 4, the AB MBIC corresponds to a several log-fold reduction in CFU/ml. For strains ATCC 35984, ATCC 12228, and X64787, the AB hystatin 3 MBIC was 64 μg/ml, which corresponded to an approximate 2 to 4 log reduction in CFU/ml. Since hystatin 3 precipitates at >64 μg/ml, it was not possible to investigate whether higher concentrations would completely eradicate the biofilms. For all strains, less than approximately 100% AB reduction was reflected in a loss of viability. These results support the hypothesis that metabolic activity is a useful measurement of viability and that the AB assay is an extremely sensitive method. Alamar blue is stable and nontoxic, so unlike other metabolic assays, it is possible to plate directly out of the wells over long periods for determination of viable cell counts. Similarly, in the NCCLS planktonic assay (17), minimum bactericidal concentrations (MBCs) are obtained from the same wells as MICs.
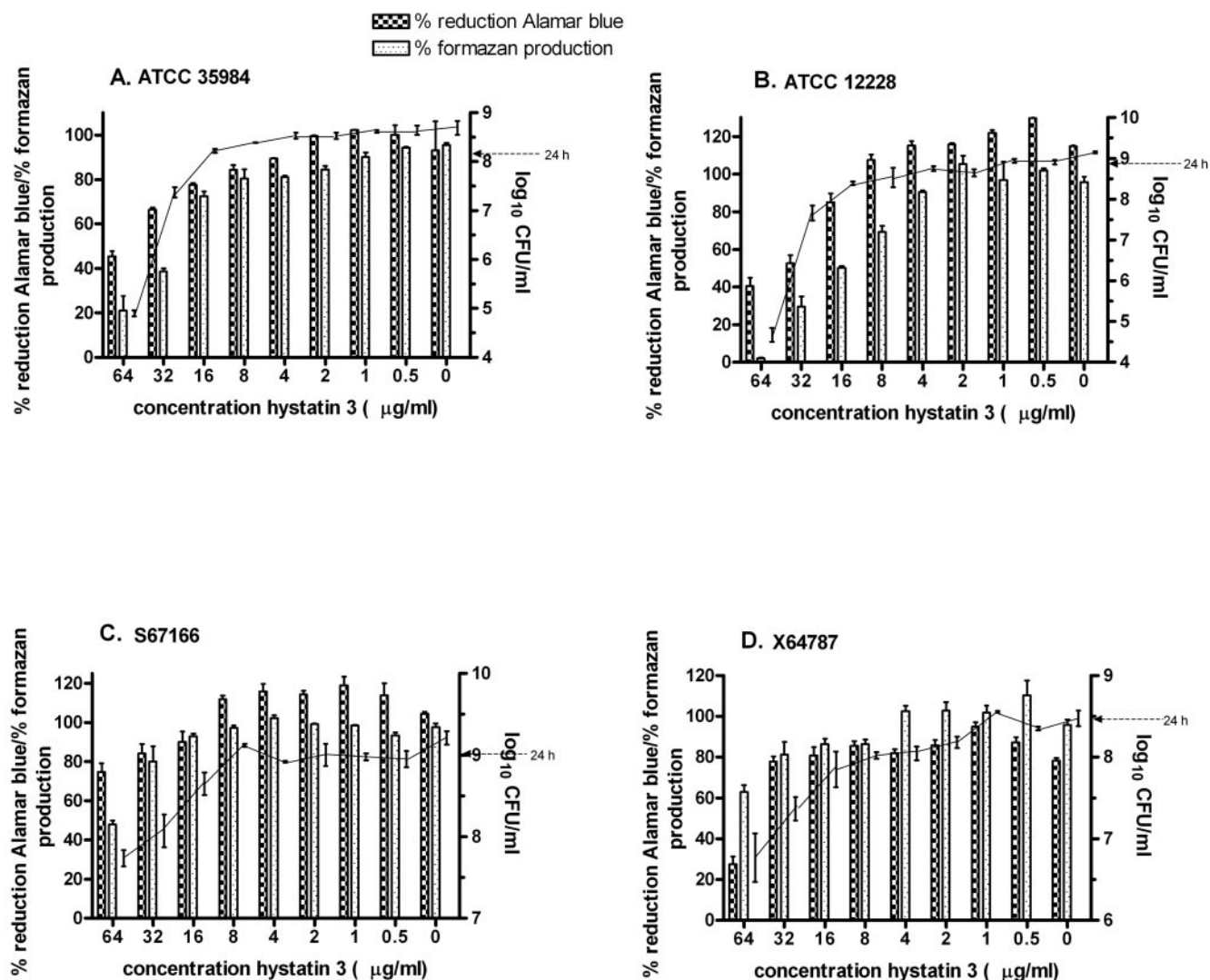

FIG. 4. Percent reduction of Alamar blue, percent formazan production, and CFU/ml (right *y* axis) for biofilms from four *S. epidermidis* strains (A-D) treated for 20 h with twofold dilutions of hystatin 3. Arrow, biofilm CFU/ml at 24 h (prior to drug treatment).

TABLE 2. Pearson's correlation coefficients for hystatin
3-treated *S. epidermidis*

S. epidermidis strain	Pearson's correlation coefficient (r) with:	
	Alamar blue vs. XTT	Alamar blue vs. CFU/ml
ATCC 35984	0.96	0.92
ATCC 12228	0.97	0.85
S67166	0.85	0.94
X64787	0.83	0.9

Pearson's two-tailed correlation coefficients were calculated from the data in Fig. 4. For all four strains, AB had good to excellent correlation with XTT and CFU/ml (Table 2), suggesting that AB deserves further evaluation as a potential standardized method of biofilm susceptibility testing.

Conclusions. The colorimetric AB assay is a reliable, reproducible means of determining biofilm antibiotic susceptibility for a major gram-positive biofilm former, *S. epidermidis*. The AB assay had good to excellent correlation with two other biofilm susceptibility methods, XTT reduction and viable counts. The benefits of AB over these and other methods of biofilm susceptibility are numerous and include simplicity, relative cost, compatibility with high throughput, lack of toxicity, and importantly, AB measures viability, not simply mass. The AB biofilm assay can be performed in any lab with a spectrophotometer and is an excellent choice for high-throughput labs. Heat and light sensitivity are drawbacks but can be avoided with simple precautions. The AB protocol now needs to be evaluated with other medically important biofilm formers, and clinical correlation needs to be assessed. A standardized biofilm AB assay should greatly increase the rate of discovery of biofilm-specific agents.

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